REMARKS/ARGUMENTS

Entry of the present Amendment under 37 CFR 1.116 is requested as placing the present application in condition for allowance or in better form for consideration on appeal. The claims have been amended so as to recite subject matter which is understood from the Final Office action to be allowable, as explained below.

By Final Office Action dated 5/16/05, claims 1, 3, 6, 9, 11-18, 24, 28-32 and 47-51 were rejected. Claims 1, 3, 6, 9, 11-18 and 51 were objected to. In the present amendment, claims 1, 16, 24, 32, 48 and 51 are amended. Claims 12-15 are cancelled. Claims 1, 3, 6, 9, 11, 16-18, 24, 28-32 and 47-54 remain pending.

Claim Amendments

The present claim amendments are intended to address the rejections of the previous Office Action, dated 5/16/2005 and are made in order to advance Applicants' business interest in obtaining an issued patent. Accordingly, a sincere effort has been made to comply with the requirements of the Office Action.

Claim 1 has been amended to incorporate limitations from claims indicated as being allowable (See reference to claims 11 and 16-18 in the Office Action). Method of treatment claim 24 recites a broader list of bacteria than that recited in claim 1. However, claim 24 has been rewritten to recite that "both said pathogenic bacteria and said attenuated bacteria are from one member of the group." Support for this limitation is found, for example, at p. 20, second portion of paragraph [0080]. See also the list at paragraph [0088]. In the examples, the vaccines administered are from homologous organisms, except that cross protection between different species of the same genus is demonstrated experimentally. Example 2 shows protection by dam- S. typhimurium against wild type S. typhimurium. Example 3 shows protection by various species of Salmonela against challenges with other species of Salmonella. Example 5 relates to protection against S.

etneritidis by immunization with that species. Example 6 relates to immunization with V. cholerae against cholera (V. cholerae) infection.

Thus there is support in the specification for the limitation that the immunizing bacteria be of the same genus as the challenge bacteria. Support for changing "treating" to "protecting against" may be found in Example 2A at page 73 of the specification. Support for other claims amendments may be found in already presented claims, in particular the bacteria now listed.

Turning now to the Detailed Action, for the convenience of the Examiner, paragraphs of the Detailed Action are set forth and responded to in the same order as presented in the Office Action.

Objections/Rejections Maintained **(Office Action Paragraph 2, page 2)**

Applicants note with appreciation the indication of allowability of claims 11 and 16-18 if rewritten in independent form. Claim 11 is currently dependent from claim 1, reciting a method of preparing a composition and is limited to certain species of bacteria. Claim 1 has been amended to incorporate the limitations of the dependent claims 16-18, namely the pathogenic bacteria listed therein.

Claim Objections/Rejections Withdrawn **(Office Action Page 2, Paragraphs 3-6)**

Withdrawal of the rejections noted in the Office Action is noted with appreciation.

New Claim Limitations/New Grounds of Rejection
Claim Objections (Office Action Page 3, Paragraphs 3-5)

The phrase "adding to it" was objected to in claim 51. Semicolons were objected to in claims 48 and 50.

Response

The "it" in "adding to it" has been specified in claim 51. The semicolons have been replaced with commas in claim 48. No objectionable semicolons could be found in claim 50.

Claim Rejections – 35 USC §112 (Office Action page 3, paragraphs 6-14)

Claims 24, 28-31, 32, 48, 50 were rejected for failure to comply with the enablement requirement. The Office Action states that "No common antigens or molecular relationship is required for the administered attenuated bacteria relative to the pathogenic bacteria causing infection." As elaborated in Office Action Paragraphs 12, and 13, the claims are interpreted to cover the use of any bacteria for fighting any infection. Further, in paragraph 9, Klem et al. is cited to show that one bacterium would not serve to competitively displace another bacteria. In paragraph 10, it is asserted that vaccines do not predictably produce the desired protection, and literature relating to H. pylori is cited. Paragraph 11 points out that all bacteria do not cause infection at the same location.

With regard to paragraph 13 of the Office Action, the method of treatment claims have been amended to specify protection against infection, which is what in fact was experimentally demonstrated.

In Paragraph 14, Amended claims 1,3,6,9, 11-18, 47, 49 and new claim 51 are rejected as being indefinite due to the recitation of the limitation "excess agent."

Response

Independent claims 24, 32 and 48 have been amended to specify that "both said pathogenic bacteria infection and said attenuated bacteria are from one member of the group." Therefore, the genus of bacteria causing the recited infection is the genus of bacteria used to treat it. Applicants' have found, as reported in *Science*, that the *dam* gene may be modulated to affect the virulence of a pathogenic bacteria. That is, the "masking" of immunity by gene methylation is interrupted and the pathogenic bacteria reveals more of its natural antigens for recognition by the host immune system. See the discussion at paragraph [00270] of the specification. Thus, the finding that *dam*- bacteria have

immunogenic protective effects, yet do not cause harmful infection, and are therefore useful in methods of treatment has not been addressed at all by the prior art of record.

It is therefore submitted that the Examiner's objection that the claims do not structurally define the administered compositions, or do not require any common antigens has been overcome by amendment. As to the teachings of Klemm et al., what has been demonstrated in the present specification is protective immunity. In Example 2A, it was shown that the protective immunity lasts at least five weeks after immunization, which is after the immunizing bacteria would have been cleared from the system. Thus the issue of competitive displacement raised by the Kamm article does not render the present methods non-enabled. The fact that different bacteria may colonize different locations in the body (Office action Paragraph 11) is also not relevant to the present immune response. The unpredictability of vaccines as allegedly shown by the De Beradinis et al. and Alshahii et al. has been overcome by experimental evidence of record. Furthermore, literature can also be cited to show that Dam- mutants confer immunity. This is shown in Julio et al. "DNA Adenine Methylase is Essential for Viability and Plays a Role in the Pathogenesis of Yersinia pseudotuberculosis and Vibrio Cholerae, Infection and Immunaty, 69:7610-7165 (Dec. 2001; Low et al. "Roles of DNA Adenine Merthylation in Regulating Bacterial Gene Expression and Virulence," Infection and Immunity 69:7197-7204 (Dec. 2001), which cite the work of the present inventors.

With regard to Paragraph 14, the claims rejected as being indefinite due to the recitation of the limitation "excess agent" have been amended to remove this phrase (claims 1 and 52).

Method of Treatment Claims

Independent claims 24, 32, and 48 were rejected as non-enabled, but not rejected over art. The present amendments to these claims do not bring them within the scope of the prior art.

There is nothing in the art of record to suggest Applicants' finding, as reported in *Science*, that the *dam* gene may be modulated to affect the virulence of a pathogenic bacteria. This is particularly unexpected in view of the gene expression of many pathogenic bacteria, which differs between in vitro growth and in vivo growth. See the discussion at paragraph [00270] of the specification. Thus, the finding that *dam*- bacteria have immunogenic protective effects in vivo, yet do not cause harmful infection, and are therefore useful in methods of treatment has not been addressed at all by the prior art of record. The recited methods also involve unexpected results in that the present methods of treatment are effective in treating species other than the species of bacteria administered (See specification paragraph 00116).

Nothing in the prior art suggests that the dam-modified strains as disclosed and claimed herein would be avirulent enough to be administered to a subject in need, yet still be protective. Furthermore, experimental work described in the present specification shows that dam modulated strains are more protective than wild type strains. Applicants' research suggests that alteration of the dam gene impairs the "camouflage" mechanism of many pathogenic bacteria in avoiding host immunity.

New claims 52, 53 and 54, dependent from method of treatment claims 24, 32, and 48, respectively limit the bacteria to those corresponding to those in claims 11 and 16-18 objected to only as depending from a rejected base claim. This is done in an attempt to comply with the Examiner's requirements and to further Applicants' business interest in obtaining an issued patent, without conceding the correctness of the rejection or Applicants' right to broader claims in a subsequent application.

Rejection of Claims 1,and 9, 12-13, 47 and 49 rejected under 35 USC §102(e) as anticipated by Ritchie et al. 1986 (Office Action Page 7, Paragraph 15-16)

Response

The limitations of claims 11 and 16-18 have been incorporated into claim 1. Since these claims were not subject to this rejection, claim 1 and dependent claims 9, 12-13, 47 and 49 should now be allowable. It is noted that Ritchie et al. pertain to a mutation in the CysG locus, presumably in the dam gene of *Salmonella typhimurium*. The effects of this lack of methylation on frameshift mutation induction were studied. However, the reference does not specifically disclose mutations in the dam gene or the effects of such mutations on virulence and immunogenicity.

Rejection of Claims 1 and 47 under 35 USC §102(e) over Thune et al. 6,010,705 (Office Action Page 9, Paragraph 17)

Thune et al. was cited as disclosing a method of making a composition having reduced bacterial virulence of a pathogenic bacteria, comprising the steps of growing, contacting, separating, and adding. The contacting step involves contacting E. ictaluri with the *E. ictaluri pur* A gene.

Response

Claim 1 has been amended as stated above to obviate this rejection.

Conclusion

Applicants request that this amendment to the claims and specification be entered and the rejections of claims 1, 3, 6, 9, 11, 16-18, 24, 28-32 and 47-51 be withdrawn. It is believed that the present Amendment places the application in condition for allowance. The allowance of currently pending claims 1, 3, 6, 9, 11, 16-18, 24, 28-32 and 47-54, as well as the timely issuance of a Notice of Allowance is earnestly solicited. The Examiner

may call Applicants' attorney at the number below in the event that a telephone conference would expedite prosecution of the present case.

Respectfully submitted,

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MINIREVIEW

Roles of DNA Adenine Methylation in Regulating Bacterial Gene Expression and Virulence

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DNA methylation provides a mechanism by which additional information is imparted to DNA, and such epigenetic information can alter the timing and targeting of cellular events (47). DNA methylation occurs throughout the living world, including bacteria, plants, and mammals. Until recently, methylated DNA sequences were not detected in the fruit fly, in brewer's yeast, or in the nematode. However, analysis by Lyko and colleagues showed that *Drosophila melanogaster* does contain methylated DNA (42, 43), and thus it is possible that yeast and worms may also have it. In this review, we focus our attention on the roles of DNA methylation in regulating bacterial gene expression and virulence. Although some background information about DNA methylation is presented, we refer the reader to excellent reviews on the subject (5, 15, 28, 47, 64).

DNA methylation occurs at the C-5 or N-4 positions of cytosine and at the N-6 position of adenine and is catalyzed by enzymes known as DNA methyltransferases (MTases) (57, 59). All MTases use *S*-adenosyl methionine as a methyl donor. DNA methylation has historically been associated with DNA restriction-modification systems thought to be important in protecting cells from foreign DNAs such as transposons and viral DNAs (35, 50, 69). Restriction-modification systems contain a DNA methylase that protects host DNA sequences from restriction with their cognate restriction enzymes which digest unmodified foreign DNAs. Certain MTases, including DNA cytosine MTase (Dcm), which methylates the C-5 position of cytosine in CC(A/T)GG sequences, DNA adenine methylase (Dam), which methylates N-6 of adenine in GATC sequences, and cell cycle-regulated methylase (CcrM), which methylates the N-6 adenine of GAnTC, do not have cognate restriction enzymes associated with them (64). These methylases participate in cellular regulatory events, including those that control bacterial virulence, which are the primary focus of this review.

Dam FAMILY

Background. Based on the organization of 10 amino acid domains present in MTases, Dam is classified in the α group (Fig. 1) (46). Dam homologues are widespread among enteric bacteria, including *Escherichia coli*, *Salmonella* spp., *Serratia marcescens*, *Yersinia* spp., and *Vibrio cholerae*, *cholerae*, but are also present in disparate genera, including *Neisseria* among others (Table 1 and Fig. 1). Dam methylation is not essential for viability of *E. coli* (3); however, recent data indicate that Dam is an essential gene in *Vibrio cholerae* and *Yersinia pseudotuberculosis* (31, 45), similar to results showing that the CcrM methylase is essential in *Caulobacter crescentus*, *Brucella abortus*, *Rhizobium meliloti*, and *Agrobacterium tumefaciens* (32, 70, 73, 93). Certain α-group methylases, including the DpnII methylase, share significant sequence identity with Dam (32% for DpnII) and methylate GATC sites like Dam but are part of restriction-modification systems (Fig. 1).

Functions. Adenine methylation can alter the interactions of regulatory proteins with DNA, either by a direct steric effect or by an indirect effect on DNA structure (18, 61, 62). Initial studies with *dam* mutants showed that Dam regulates the expression of certain genes in *E. coli* including *trpR* (60), Tn*10* transposase (68), and *dnaA* (13) as well as phage genes including *mom* of Mu (24). Methylation of a GATC site(s) within the consensus RNA polymerase binding site inhibits (*trpR* and *Tn*10 transposase) or enhances (*dna*A) transcription, by altering the interaction with the transcription apparatus.

As discussed above, methylation can alter the affinity of regulatory proteins for DNA. Conversely, DNA binding proteins have been shown to inhibit methylation of specific DNA sequences. For example, the SeqA protein involved in the timing of DNA replication binds specifically to hemimethylated DNA sequences near the origin of replication (*oriC*), thereby sequestering *oriC* from Dam methylation for a part of the cell cycle and maintaining it in a hemimethylated state (41). Other regulatory proteins bind nonmethylated DNAs with highest affinity, protect specific DNA sequences from methylation, and form DNA methylation patterns (DMPs), which are present in certain eukaryotes as well (see Fig. 2) (28). DMPs are formed when regulatory factors bind to DNA target sites that overlap or are near methylation sites and inhibit their methylation (12).

Regulation by hemimethylation. Work carried out with *E. coli* Dam indicates that it acts as an efficient de novo methylase, methylating both nonmethylated and hemimethylated GATC sites with similar efficiency (82). Dam plays an important role in regulating the timing and targeting (51) of a number of cellular functions including DNA replication (9, 34, 41, 72), segregation of chromosomal DNA (52, 58), mismatch repair (29, 48, 71), and transposition (19, 66, 68, 77, 89). In all of these events, hemimethylated GATC sites, present immediately following DNA replication, control the binding of pro-

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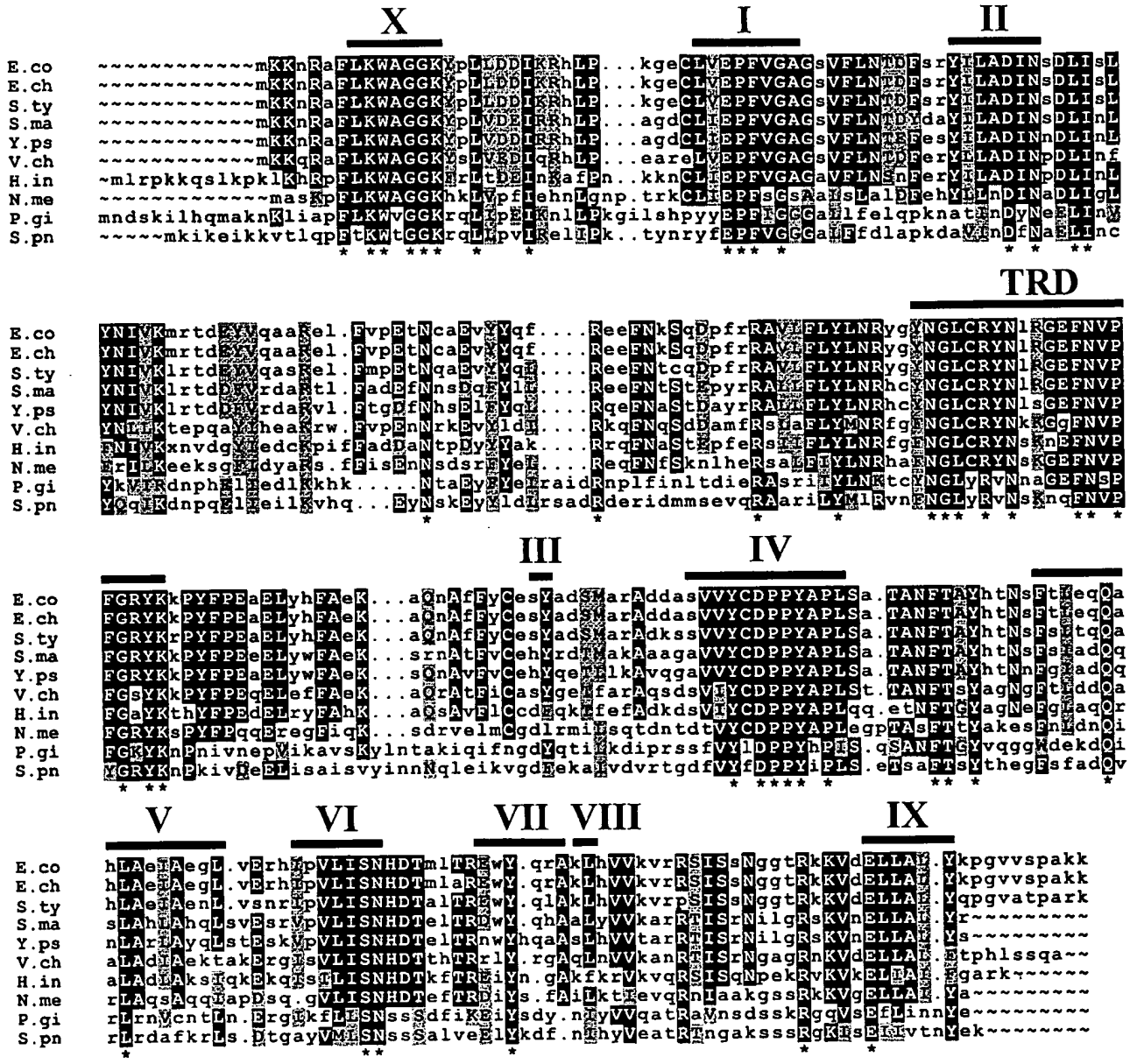

FIG. 1. Sequence alignment of selected *E. coli* Dam homologues. Residues that are identical in all 10 proteins are noted with asterisks. Residues that are identical or similar in 70% of the sequences are boxed in black and gray, respectively. Lowercase letters indicate residues that occur in less than 70% of the listed sequences. Motifs implicated in the binding of *S*-adenosyl methionine and methyl transfer are labeled with Roman numerals according to the nomenclature of Tran et al. (80; also see reference 46). The putative GATC site recognition domain of DpnM, an α family DNA adenine methylase (80), is indicated by TRD. The alignment was created using the PILEUP program of the Genetics Computer Group sequencing package (17). *E. chrysanthemi* sequences were provided by C.-H. Yang and Noel T. Keen (unpublished data). E.co, *Escherichia coli* (P00475) (SWISSPROT or GenPept database accession numbers are listed in parentheses); E.ch, *Erwinia chrysanthemi*; S.ty, *Salmonella* serovar Typhimurium (P55893); S.ma, *Serratia marcescens* (P45454); Y.ps, *Yersinia pseudotuberculosis* YPIII (AF274318); V.ch, *Vibrio cholerae* O395 (AF274317); H.in, *Haemophilus influenzae* (P44431); N.me, *Neisseria meningitidis* (AAD34292); P.gi, *Porphyromonas gingivalis* (S34414); and S.pn, *Streptococcus pneumoniae* (P04043).

teins to specific DNA target sites. For example, DNA replication is controlled in part by SeqA, which binds specifically to hemimethylated GATC sites near the origin of replication and delays their methylation (34, 41). Segregation of newly replicated DNA may occur by binding of hemimethylated DNA to membrane-bound factors. In methyl-directed mismatch repair, MutH binds to hemimethylated DNA and cleaves the non-methylated strand (1). Certain transposases, including Tn*10* transposase, bind with highest affinity to hemimethylated binding sites, limiting transposition to a time immediately following DNA replication (68).

Regulation by DNA methylation patterns. Dam also plays pivotal roles in controlling gene expression by the formation of DMPs. DMPs have long been known to be present in eu-

TABLE 1. Roles of Dam in bacterial virulence

Bacterial pathogen[a]	Methylase family	Target sequence	% identity to *E. coli* Dam[b]	Role for methylase in:		Reference
				Growth	Virulence	
Escherichia coli	Dam (α)	GATC	100	No	Yes	36
Erwinia chrysanthemi	Dam (α)	GATC	99	No	Yes	C.-H. Yang and N. Keen[c]
Salmonella enterica serovar Typhimurium	Dam (α)	GATC	92	No	Yes	21, 25, 26
Yersinia pseudotuberculosis	Dam (α)	GATC	71	Yes	Yes	31
Vibrio cholerae	Dam (α)	GATC	64	Yes	Yes	31
Neisseria meningitidis	Dam (α)	GATC	47	No	Yes	14
Brucella abortus	CerM (β)	GAnTC	NA	Yes	Yes	70

[a] The SWISSPROT or GenPept database accession numbers are listed in parentheses for the following strains: *E. coli* (P00475), *Salmonella* serovar Typhimurium (P55893), *Yersinia pseudotuberculosis* YPIII (AF274318), *Vibrio cholerae* O395 (AF274317), *Haemophilus influenzae* (P44431), and *Neisseria meningitidis* (AAD34292).
[b] Percent identities among the selected *E. coli* Dam homologs were calculated using the GAP program of the Genetics Computer Group sequencing package (17). NA, not applicable.
[c] Yang and Keen, unpublished data.

karyotes and appear to regulate gene expression (5, 92). The first reported DMPs which regulate gene expression in prokaryotes are within the pyelonephritis-associated pilus (*pap*) operon of uropathogenic *E. coli* (7).

Most nonmethylated GATC sites are found within noncoding regions that likely have a regulatory function. For example, the GutR repressor binds in the upstream regulatory region of the glucitol (*gut*) operon, blocking methylation of a GATC site designated GATC −44.5 within the GutR binding domain and forming a specific DMP (85). In the presence of glucitol, GutR no longer blocks DNA methylation, indicating that it no longer binds *gut* regulatory DNA. This is an example of environmental control of a DMP. Notably, although catabolite gene activator protein (CAP) also binds to a site overlapping GATC −44.5, it does not protect this site from methylation.

It appears that most, if not all, DMPs are formed by the binding of regulatory proteins such as GutR (85), Lrp (12), histone-like nucleoid-structuring protein (H-NS) (91), and OxyR (22) to upstream regulatory DNA sequences. In these examples, purified proteins have been shown to block methylation of GATC sites that are contained in or near the DNA recognition sequence in vitro. Inhibition of methylation could occur by direct steric occlusion of Dam binding or by alterations in DNA conformation which change the configuration of the Dam target (GATC) site (61).

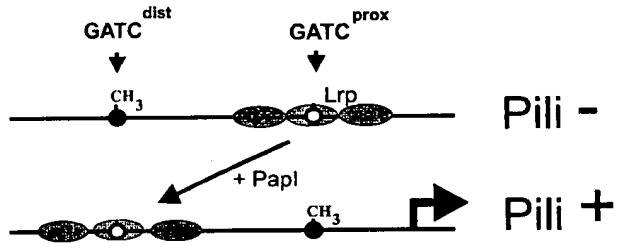

FIG. 2. Regulation of Pap pilus expression by DNA methylation patterns. Cooperative binding of Lrp to sites proximal to the *papBA* pilin promoter blocks transcription, inhibits methylation of GATC^{prox}, and forms the phase-OFF DNA methylation pattern (Pili$^-$ state) (90). According to the Pap phase variation model (see the text) (36, 84), transition to the Pili$^+$ state occurs following DNA replication under conditions which induce PapI expression. PapI binds specifically to Lrp, increases the affinity of Lrp for distal *pap* DNA binding sites, and forms the phase-ON DNA methylation pattern (33, 55, 56).

Analysis of the *E. coli* chromosome has shown that there are at least 50 GATC sites that are stably undermethylated (67, 75). The locations of these sites may vary depending upon environmental conditions (23), which can alter the expression and/or binding of regulatory factors that bind to DNA and specifically block DNA methylation. Analysis of the *Salmonella* chromosome by pulsed-field gel electrophoresis has shown that, similar to *E. coli*, specific DNA methylation patterns are present (26). Although many nonmethylated GATC sites have been identified in *E. coli*, methylation in only a subset has been shown to control the expression of linked genes. For example, GutR blocks methylation of GATC −44.5 in the *gut* operon, but methylation of GATC −44.5 does not alter the binding of GutR to *gut* DNA in vitro nor does it alter *gut* expression in vivo (85). The CarP regulatory protein and integration host factor (IHF) protect a GATC site in the *carAB* operon, but it is not clear if methylation controls CarAB expression (16). In contrast, methylation of regulatory GATC sites in the *pap* and *agn* operons directly controls expression of Pap pili and the Ag43 outer membrane protein, respectively (12, 22, 27). This occurs by reduction of the affinities of the Lrp and OxyR proteins for *pap* and *agn* regulatory DNAs, respectively. In *pap* and related methylation-controlled operons, methylation of two GATC sites spaced 102 bp apart regulates Lrp binding, whereas in *agn*, methylation of three closely spaced GATC sites inhibits OxyR binding (22). In these instances, there is a mutual competition between the methylase and the DNA binding protein, forming a DMP which heritably controls gene expression and provides a form of cellular memory.

ROLES OF Dam IN VIRULENCE

Alterations in the levels of Dam attenuate the virulence of a number of pathogens, including *Salmonella* spp. (21, 25, 26), *Y. pseudotuberculosis*, and *V. cholerae* (31). Because Dam plays multiple roles in cell physiology (see above), it is possible that pleiotropic effects not related to alterations in gene expression may be responsible for the virulence defects of *dam* mutants. However, the growth rates of *dam* mutant and Dam-overproducer *Salmonella enterica* serovar Typhimurium were similar to that of wild-type *Salmonella* (25). In addition, levels of overproduction of Dam in *Y. pseudotuberculosis* and *V. cholerae* that inhibited virulence had no significant effect on in vitro growth rates (31). These data strongly indicate that the viru-

lence defect of *dam* mutants is directly the result of alterations in gene expression and not due to a nonspecific growth defect. Dam has been reported to control the expression of a number of virulence genes (12, 21, 25, 26, 31, 44, 45). Deletion of *dam* erases DNA methylation patterns, which could alter the binding of regulatory proteins to a number of regions on the bacterial chromosome. In the absence of Dam, overexpression of genes could occur if GATC methylation blocked binding of an activator or enhanced the binding of a repressor. Conversely, underexpression of a gene would occur in the absence of Dam if GATC methylation blocked binding of a repressor or enhanced binding of an activator.

E. coli. Dam regulates the expression of a large group of pilus operons that play important roles in virulence in urinary tract infections (e.g., Pap, Prf, and S pili) and diarrheal diseases (e.g., Afa, CS31a, and K88 pili) (30, 36, 49, 53, 87). All of these pilus-adhesin operons share common regulatory features including control by Lrp, the presence of a promoter proximal GATC site ($GATC^{prox}$) located within one set of Lrp binding sites and a promoter distal site ($GATC^{dist}$) within a second set of Lrp binding sites, inter-GATC spacing of 102 or 103 bp, and a homologue to the PapI regulatory protein that binds to Lrp, increasing its affinity for the promoter distal Lrp sites which then helps activate transcription (7, 8, 11, 12, 33, 55, 56, 86) (Fig. 2).

In the *pap* operon, DNA methylation directly regulates the switch between pilus expression (phase ON) and nonexpression (phase OFF) by dictating the binding of Lrp (Fig. 2). At low PapI levels, Lrp binds with high affinity to promoter proximal sites, blocks transcription from the *papBA* promoter, and inhibits methylation of $GATC^{prox}$ (90). However, the promoter distal $GATC^{dist}$ site is not bound by Lrp and thus becomes methylated. Methylation of $GATC^{dist}$ inhibits movement of Lrp to the distal set of sites and thus is presumed to lock cells in the phase-OFF state until DNA replication generates a hemimethylated $GATC^{dist}$ site which binds Lrp with a higher affinity (36). Methylation of the promoter proximal $GATC^{prox}$ site is required for the expression of Pap pili (12). Mutations in the Lrp binding sites near $GATC^{prox}$ result in a phase-locked ON transcription phenotype that is Dam and PapI independent (55). These results indicate that methylation of $GATC^{prox}$ may help displace Lrp from its promoter proximal DNA binding sites that overlap $GATC^{prox}$, with the aid of PapI (33). Binding of Lrp-PapI at the promoter distal $GATC^{dist}$ site blocks its methylation, forming a DNA methylation pattern that is characteristic of cells expressing pili. The cell environment controls the *pap* DNA methylation pattern since in poor carbon sources the cyclic AMP level is high and stimulates PapI expression via a cyclic AMP-CAP binding site in the *pap* regulatory region (2). PapI facilitates movement of Lrp to the $GATC^{dist}$ site, and Lrp blocks methylation of $GATC^{dist}$ and helps activate *pap* transcription (84). Other members of the Pap family, including *sfa* (87), *daa* (87), *fae* (30), and *clp* (49) and *pef* in *Salmonella* serovar Typhimurium (53), have been shown to be regulated by DNA methylation patterns as well (36).

Salmonella. Torreblanca and Casadesus first identified genes regulated by Dam in *Salmonella* serovar Typhimurium using a genetic approach (78). One of these genes mapped to the pSLT virulence plasmid and was later shown to be *finP,* which expresses an antisense RNA controlling the F-type pili required for conjugative plasmid transfer (79). The result is that under conditions of low levels of Dam, transfer of the *Salmonella* pSLT plasmid is elevated. The physiologic connection between Dam-controlled pilus expression and mating is not yet clear but could function to coordinate mating with virulence plasmid replication or to enable environmental control of mating (79). Dam also regulates the expression of plasmid-encoded fimbriae (Pef) encoded by pSLT by a mechanism that shares features with *pap* (53). Work from Heffron's laboratory indicates that Pef may play a role in *Salmonella* virulence (83). Like *pap*, expression of Pef fimbriae is turned off in the absence of Dam since methylation of a promoter-proximal GATC site of *pef* is essential for transcription.

Recently, Dam was shown to be essential for the virulence of *Salmonella* serovar Typhimurium in a murine model of typhoid fever (21, 25, 26). Dam^- *Salmonella* shows reduced M-cell cytotoxicity and invasion of enterocytes but appears to grow normally within cells (21). In the absence of Dam, serovar Typhimurium is avirulent when given orally and intraperitoneally and fails to kill mice at 10,000 times the lethal dose required to kill half of the animals (LD_{50}). Dam-deficient *Salmonella* colonizes Peyer's patches in a manner similar to that of wild-type bacteria but attains only very low numbers in systemic tissues and is totally cleared from mice after about 4 weeks. The failure of *dam* mutants to cause disease is not the result of defects in mismatch repair since *mutS* and *mutL* serovar Typhimurium is fully virulent (21, 26).

Why is Dam^- *Salmonella* avirulent? We hypothesize that *dam* mutant *Salmonella* is markedly attenuated as a result of dysregulation of gene expression. Dam-deficient *Salmonella* serovar Typhimurium up-regulates the expression of over 35 genes that are induced during infection (26), including *spvB*, a cytotoxin which causes apoptosis of macrophages (39). In contrast, Dam positively regulates the secretion of the SipA, SipB, and SipC proteins coded for by the Salmonella pathogenicity island type 1 virulence locus (21). Thus, in the absence of Dam, virulence factors such as SpvB are predicted to be overexpressed (the SpvB protein was recently shown to be ectopically expressed at very high levels in the absence of Dam [D Guiney, unpublished results]), whereas other factors such as SipABC are underexpressed. We hypothesize that this combination of overexpression and underexpression of virulence proteins inhibits virulence (25, 45). If this is correct, then overexpression of Dam might also block virulence since negatively regulated factors such as SpvB would be underexpressed and positively regulated factors such as SipABC would be overexpressed. In fact, overexpression of Dam reduces the virulence of *Salmonella* serovar Typhimurium 10,000-fold (25). As predicted, the protein profiles from Dam^- and Dam-overproducing *Salmonella* strains are different from each other and from wild-type *Salmonella* (25). In addition, Dam^- *Salmonella* releases a high level of outer membrane vesicles, suggesting an instability defect in the outer membrane. Since OmpA is a highly immunogenic protein, vesicle release by *dam* mutants might contribute to their efficacy as live attenuated vaccines (J. Casadesus, personal communication). These data support the hypothesis that Dam is a global regulator of virulence genes in *Salmonella* and that Dam levels regulate virulence.

Both Dam-deficient and Dam-overproducing *Salmonella* se-

rovar Typhimurium strains are highly effective vaccines against salmonellosis. As few as 90 Dam$^-$ bacteria administered intraperitoneally provide significant protection (21) and oral vaccination of mice with 10^9 Dam$^-$ *Salmonella* bacteria completely protects them from challenge with 10^9 wild-type *Salmonella* bacteria (10,000 times the oral LD_{50}) (26). Vaccination with Dam$^-$ *Salmonella* protects mice against challenge with other *S. enterica* serovars including Enteritidis and Dublin (25). Additionally, Dam$^-$ *Salmonella* conferred cross-protective immunity in chickens (20a). This cross-protection could occur as a result of aberrant expression of a number of virulence determinants of serovar Typhimurium, some of which might be shared with other *Salmonella* serovars. The dysregulation of expression of *Salmonella* virulence determinants not only could disrupt the normal pathogenic cycle but also may enable the host immune system to mount an effective response. This response could be elicited to Dam-controlled bacterial antigens which are normally under temporal and spatial control and not easily detected. Consistent with this hypothesis, *S. enterica* serovar Typhimurium overproducing Dam conferred significant protection against homologous *Salmonella* (25) although not to the same extent as Dam-deficient *Salmonella*. This discrepancy could be due, in part, to the differences in antigen expression observed between these two vaccines (see above). Dam is also essential for full virulence of *S. enterica* serovar Enteritidis (25), which can invade the yolk sac and contaminate eggs. Because Dam$^-$ *S. enterica* serovars Typhimurium and Enteritidis are highly attenuated, it seems likely that Dam might also be essential for virulence of *S. enterica* serovar Typhi, the causative agent of typhoid fever.

Other pathogens. Although Dam from *E. coli* and *Salmonella* spp. is not essential for growth, *dam* is an essential gene in *V. cholerae* and *Y. pseudotuberculosis* (31) (Table 1). Since *V. cholerae* has two chromosomes (81), it is possible that Dam plays the same roles as those in *E. coli* and *Salmonella*, but additionally, Dam may coordinate the timing and segregation of the two chromosomes. Recent data indicate that the virulence of Dam$^-$ *Erwinia chrysanthemi* is greatly reduced for African violets and lettuce, two of its hosts (C.-H. Yang and N. Keen, unpublished data). Thus, Dam is important for the virulence of both animal and plant pathogens.

Overproduction of Dam inhibits the colonization of *V. cholerae* based on a suckling mouse model (31). Moreover, overexpression of Dam in *Y. pseudotuberculosis* greatly attenuates virulence in a murine model (>6,000-fold) and alters the protein expression profile. Oral immunization of mice with Dam-overproducing *Y. pseudotuberculosis* protects against challenge with at least 1,000 times the LD_{50}. Thus, it appears that the Dam-based vaccination strategy developed using *S. enterica* serovar Typhimurium can be extended to other enteric pathogens.

In contrast to the essentiality of Dam for virulence and/or growth in enteric bacteria, all pathogenic isolates of group B *Neisseria meningitidis* have a mutation within *dam* (*dam* gene replacement, or *drg*) and thus do not express Dam (14). These meningococci are deficient in methyl-directed mismatch repair, as expected, and show high rates of phase variation of a neuraminic acid capsule controlled by the polysialyltransferase (*siaD*) gene. Frameshift mutations within a poly-deoxycytosine repeat in the *siaD* gene coding sequence regulate the on-off expression of capsule. A high rate of capsular phase variation was observed only in *dam* mutant meningococci. Thus, selection for Dam-deficient meningococci may occur as a result of increased phase variation rates.

We are only beginning to understand the roles that Dam plays in regulating the interactions between bacterial pathogens and their hosts that contribute to virulence. Many bacterial pathogens, including *S. enterica* serovar Typhi, *Shigella* spp. (dysentery), pathogenic *E. coli* including O157:H7 (hemolytic-uremic syndrome), *Haemophilus influenzae* (pneumonia and otitis media), and *Legionella pneumophila* (pneumonia), have been reported to contain *dam* homologues and/or Dam activity (25, 38, 45) (Table 1 and Fig. 1). It seems likely that Dam is important for the pathogenesis of a number of diseases. An important next step will be to determine the mechanism by which Dam controls virulence and whether there are any unifying concepts that can be gleaned from the analysis of genes regulated by Dam in a number of pathogens.

Alteration in DMPs caused by fluctuations in the binding of regulatory proteins such as Lrp or OxyR to DNA target sites can be viewed as a novel mechanism for global regulation. The relevance of DMPs to pathogenesis could be determined by identification of all Dam-regulated virulence genes in a pathogen by microarray analysis and matching them to the nonmethylated GATC sites in the chromosome (75, 88). The first part of this analysis has recently been carried out in *E. coli*, with the data indicating that the mRNA levels of about 50 to 60 genes are significantly altered in the absence of Dam (N. Bourquard, G. W. Hatfield, and D. A. Low, unpublished data).

CcrM FAMILY

Background. In contrast to Dam, CcrM is classified in the β group of DNA methylases (Table 1). CcrM was originally discovered as a cell cycle-regulated MTase in *Caulobacter* (95). CcrM is 49% identical to the *Hin*fI MTase from *H. influenzae*, which shares the same recognition sequence but is part of a retriction-modification system. CcrM homologues are found in the α-proteobacteria, including the plant pathogen *A. tumefaciens* and symbiont *Rhizobium meliloti* and *B. abortus* (64), which causes brucellosis in cattle and humans (70). All CcrM homologues tested appear to be essential for cell viability, similar to the essential roles of MTases in mammalian cells and Dam in *V. cholerae* and *Y. pseudotuberculosis*. Moreover, based on complementation analysis of *Caulobacter* and *Rhizobium*, the CcrM proteins are functionally interchangeable (64).

Functions. The CcrM methylase from *C. crescentus* plays an essential role in the cell cycle of this developmentally programmed bacterium. CcrM, in contrast to Dam, appears to be a "maintenance" methylase with preference for hemimethylated DNA over nonmethylated DNA (4). CcrM methylase is essential for the viability of α-proteobacteria including *C. crescentus, B. abortus, R. meliloti*, and *A. tumefaciens* (32, 70, 73, 93).

Initial evidence that CcrM regulates gene expression came from analysis of the *ccrM* gene itself. Methylation of GAnTC sites within the *ccrM* promoter inhibits transcription. Mutation of these CcrM target sites prevents shutdown of methylase gene transcription after cell division (74). Analysis of the complete genome sequence of *C. crescentus* showed that CcrM

target sites were less abundant than predicted from random occurrence and had a bias to intergenic regions (54). These data support the hypothesis that CcrM is a global regulator of gene expression. Indeed, recent microarray analysis by Lucy Shapiro and colleagues indicates that approximately 100 genes in *C. crescentus* are affected by depletion of CcrM (L. Shapiro, unpublished data).

Roles in virulence. CcrM may play an important role in the virulence of *B. abortus* based on the analysis of bacteria overexpressing the methylase (70). Overexpression of CcrM on recombinant plasmids inhibited growth of *Brucella* within murine peritoneal macrophages. This attenuation did not appear to be due to alterations in bacterial growth rates or alterations in cell morphology or DNA replication initiation at lower CcrM expression levels. It thus appears that the defect in intracellular replication is due to some other effect of CcrM on cellular function such as regulation of a gene(s) required for adapting to the intracellular environment (64).

REGULATION OF DNA METHYLATION

Dam and CcrM methylase activities are under complex regulatory control, as expected for global regulators. CcrM transcription is activated by the cell cycle transcription regulator (CtrA) in late S phase in predivisional cells, resulting in fully methylated chromosomes which initiate replication. By the time cell division occurs, the CcrM level is greatly reduced via cleavage with Lon protease (65). Thus, both the level of CcrM and its cellular location in the *Caulobacter* morphogenic pathway are strictly regulated. In addition, *ccrM* may be under autoregulatory control since methylation of two CcrM target sites in the *ccrM* promoter may play a role in inhibiting *ccrM* transcription (74).

There are only about 130 molecules of Dam in rapidly growing cells, a number sufficient to methylate all of the available GATC sites within a single DNA replication cycle (10). *dam* methylase from *E. coli* contains five promoters, with the major promoter (P2) located about 3.5 kbp upstream of the *dam* AUG translation start site (40). The *dam* P2 promoter is controlled by growth rate, with high levels of *dam* transcription present in cells with high growth rates (63). The functions of the other *dam* promoters are unknown but may be responsive to in vivo growth conditions. Precedent for this possibility comes from the analysis of *Helicobacter pylori*, the causative agent of chronic gastritis (6). *H. pylori* contains a number of putative methylases without cognate restriction enzymes, and thus it has been hypothesized that these methylases may control cellular functions by analogy with CcrM and Dam (76). One *Helicobacter* gene, *hpyIM* (GenBank accession number AAC45818), codes for an adenine methylase which recognizes the sequence CATG (20, 94). HpyIM appears to be a member of the α group of methylases (46), sharing 61 and 34% identity with *Nla*III methylase (Swiss Prot accession number P24582) and a *Campylobacter* methylase (EMBL accession number CAB72691), respectively. Notably, *hpyIM* expression appears to be induced following attachment of *H. pylori* to gastric epithelial cells, suggesting that induction of HpyIM in the host may play a role in the regulation of virulence (37).

CONCLUSIONS

Clearly, DNA methylation plays important roles in the virulence of a growing list of bacterial pathogens (Table 1). DNA methylation provides an additional level of regulatory control since the binding of many different regulatory factors to target DNA sequences can potentially be affected in a heritable fashion. Moreover, alteration of methylase levels in response to environmental stimuli could control the temporal expression of specific gene subgroups depending on the effect of methylation on the affinity of each regulatory protein for target DNA. Important questions for future research include the following: What is the spectrum of pathogens in which DNA methylation plays a role(s) in virulence? What types of virulence genes are regulated by DNA methylation? What mechanisms are involved in controlling and coordinating virulence gene expression by DNA methylation? How are DNA methylase levels altered in response to environmental stimuli? Does methylation provide a memory system to help bacterial pathogens time and coordinate the expression of virulence determinants?

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DNA Adenine Methylase Is Essential for Viability and Plays a Role in the Pathogenesis of *Yersinia pseudotuberculosis* and *Vibrio cholerae*

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***Salmonella* strains that lack or overproduce DNA adenine methylase (Dam) elicit a protective immune response to different *Salmonella* species. To generate vaccines against other bacterial pathogens, the *dam* genes of *Yersinia pseudotuberculosis* and *Vibrio cholerae* were disrupted but found to be essential for viability. Overproduction of Dam significantly attenuated the virulence of these two pathogens, leading to, in *Yersinia*, the ectopic secretion of virulence proteins (*Yersinia* outer proteins) and a fully protective immune response in vaccinated hosts. Dysregulation of Dam activity may provide a means for the development of vaccines against varied bacterial pathogens.**

Salmonella DNA adenine methylase (Dam) mutants ectopically express multiple genes that are normally induced during infection (18, 20, 27). These Dam mutants are markedly attenuated but highly effective as live vaccines against murine typhoid fever (12, 20). DNA adenine methylases are highly conserved in many pathogens such as *Vibrio cholerae* (http://www.tigr.org), *Salmonella enterica* serovar Typhi (http://www.sanger.ac.uk), pathogenic *Escherichia coli* (2), *Yersinia pestis* (http://www.tigr.org), and *Haemophilus influenzae* (10). The goal of this study was to determine whether the findings regarding Dam's role in *Salmonella* pathogenesis could be extended to *V. cholerae* and *Yersinia pseudotuberculosis*, the causative agents of human cholera and gastroenteritis, respectively; additionally, *Y. pseudotuberculosis* causes a fatal bacteremia in mice. In contrast to *Salmonella*, which is a facultative intracellular parasite, both *Y. pseudotuberculosis* and *V. cholerae* are principally extracellular pathogens. *Yersinia* sp. pathogenesis is dependent upon virulence proteins called Yops (for Yersinia outer proteins) (6, 15, 37), which, upon host contact, are injected directly into the host cell cytoplasm, where they act as effectors to inhibit phagocytosis and proinflammatory cytokine release (31, 32, 36, 38). In contrast, *V. cholerae* is a mucosal pathogen that expresses virulence factors, including cholera toxin and toxin coregulated pilus, in the small intestine (9).

In this report, we show that Dam is essential for the viability of *Y. pseudotuberculosis* and *V. cholerae*. Overproduction of Dam was not lethal and attenuated the virulence of both pathogens. Additionally, Dam-overproducing strains of *Yersinia* ectopically secreted Yop virulence proteins in vitro and conferred full protection against *Yersinia* bacteremia in vaccinated hosts.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Derivatives of *Y. pseudotuberculosis* strain YPIIIpYV (obtained from Stanley Falkow) and *V. cholerae* strain 0395 (classical, Ogawa serotype; obtained from John Mekalanos) (Table 1) were grown overnight with shaking at 28 and 37°C, respectively. The following antibiotics were used at the indicated concentrations: for *V. cholerae*, kanamycin (50 μg/ml), ampicillin (50 μg/ml), tetracycline (1.2 μg/ml), and streptomycin (100 μg/ml); and for *Y. pseudotuberculosis*, kanamycin (50 μg/ml), ampicillin (50 μg/ml), tetracycline (5 μg/ml), and chloramphenicol (20 μg/ml).

Construction of genomic DNA libraries. Genomic DNA libraries for *Y. pseudotuberculosis* and *V. cholerae* were constructed using approximately 2 to 5 μg of genomic DNA that was partially digested with *Sau*3AI and size fractionated to 2.5 to 6 kb on a 0.8% agarose gel. The size-fractionated DNA was ligated into the vector pWKS30 (ampicillin resistant [Ap^r] [43]) that was previously cleaved with *Bam*HI. The recombinant plasmids were introduced into *E. coli* DH5α λ*pir* by electroporation; we then pooled the Ap^r recombinant clones, from which plasmid DNA was isolated.

Construction of plasmids containing *dam* derived from *V. cholerae*, *Y. pseudotuberculosis*, and *E. coli*. Plasmid pWKS30Tc is a tetracycline-resistant derivative of pWKS30 (43). The *tet* gene and promoter from pBR322 were excised as an *Ava*I/*Eco*RI fragment, treated with the Klenow fragment to produce blunt ends, and cloned into plasmid pWKS30, which had been partially digested with *Bgl*II and *Ssp*I (removing the *bla* gene) and treated with the Klenow fragment to produce pWKS30Tc. The *V. cholerae dam* gene was cloned into the blunt-ended *Sma*I site of pWKS30Tc by ligating a 1.2-kb *Dra*III/*Eco*RI chromosomal DNA fragment containing *V. cholerae dam* that had been treated with the Klenow fragment, generating a pWKS30Tc plasmid harboring *V. cholerae dam*. The *Y. pseudotuberculosis dam* gene was cloned into the blunt-ended *Sma*I site of pWKS30Tc by ligating a 1.3-kb *Nco*I/*Pvu*II chromosomal DNA fragment containing *Y. pseudotuberculosis dam* that had been treated with the Klenow fragment, generating a pWKS30Tc plasmid harboring *Y. pseudotuberculosis dam*. The *E. coli dam* gene was cloned into the blunt-ended *Sma*I site of pWKS30Tc by ligating a 1.3-kb *Xba*I/*Pvu*II chromosomal DNA fragment containing *E. coli dam* that had been treated with the Klenow fragment, generating a pWKS30Tc plasmid harboring *E. coli dam*. Plasmid clones containing the putative *V. cholerae*, *Y. pseudotuberculosis*, and *E. coli dam* genes were introduced into an *E. coli dam* mutant strain. Recovered plasmids were found to be resistant to the methylation-sensitive restriction enzyme *Mbo*I, indicating that the recombinant clones encode Dam.

Plasmid pTP166Cm is a chloramphenicol-resistant derivative of pTP166 (Ap^r), which overproduces *E. coli dam* from a P_{tac} promoter (29). A blunt-ended 1.4-kb *Bsa*AI fragment containing the chloramphenicol resistance gene and its promoter from pACYC184 was ligated to a pTP166 plasmid derivative that had been digested with *Dra*I and *Aat*II (removing the *bla* gene) and treated with the Klenow fragment. The resulting chloramphenicol-resistant clone, pTP166Cm, overproduced *E. coli dam*, as evidenced by the DNA methylase assay (see below).

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
Strains		
Y. pseudotuberculosis		
YPIIIpYV	Wild type	Stanley Falkow
MT2294	*dam*::Kn + pTP166-Cm	This work
MT2357	DUP[ϕ(*dam*::Kn)*pCVD442*(*dam*$^+$)]	This work
MT2358	DUP[ϕ(*dam*::Kn)*pCVD442*(*dam*$^+$)] pWKS30-Tc + *Y. pseudotuberculosis dam*$^+$	This work
MT2359	DUP[ϕ(*dam*::Kn)*pCVD442*(*dam*$^+$)] pWKS30-Tc + *E. coli dam*$^+$	This work
MT2360	DUP[ϕ(*dam*::Kn)*pCVD442*(*dam*$^+$)] pWKS30-Tc	This work
V. cholerae		
O395	Wild type; classical	John Mekalanos
MT2284	*dam*::Kn pWKS30-Tc + *E. coli dam*$^+$	This work
MT2361	DUP[ϕ(*dam*::Kn)*pCVD442*(*dam*$^+$)]	This work
MT2362	DUP[ϕ(*dam*::Kn)*pCVD442*(*dam*$^+$)] pWKS30-Tc + *V. cholerae dam*$^+$	This work
MT2363	DUP[ϕ(*dam*::Kn)*pCVD442*(*dam*$^+$)] pWKS30-Tc + *E. coli dam*$^+$	This work
MT2364	DUP[ϕ(*dam*::Kn)*pCVD442*(*dam*$^+$)] pWKS30-Tc	This work
S. enterica serovar Typhimurium		
MT2188	*damΔ232*	20
Plasmids		
pTP166-Cm	*E. coli dam* under *tac* promoter control; chloramphenicol-resistant (Ap^s) derivative of pTP166	29; this work
pWKS30-Tc	Tetracycline-resistant (Ap^s) derivative of pWKS30	43; this work

DNA sequencing and protein sequence analysis. The nucleotide sequences of the *dam* genes were determined using a Big Dye Terminator reaction kit, and samples were analyzed on a model 310 genetic analyzer (PE Biosystems). Protein sequence alignment was performed using the CLUSTAL W method available at the European Bioinformatics website (http://www2.ebi.ac.uk/clustalw/).

DNA adenine methylase assays. Dam activity was measured by a modified tritiated *S*-adenosylmethionine ([^{3}H]SAM) incorporation assay (13) wherein SAM serves as a methyl donor for DNA methylases. Briefly, cells from an overnight grown culture (10 ml) were collected by centrifugation, quick frozen under liquid nitrogen, washed once in 1× Tris-EDTA buffer (10 mM Tris HCl [pH 8.0]–1 mM EDTA [pH 8.0]), and resuspended in 10× Tris-EDTA. Lysozyme (0.05 mg) was added, and the cells were disrupted by sonication. Cell debris was removed by centrifugation, and the cell extracts were recovered. The total amount of protein in each cell extract was determined by a Bradford protein assay. GATC-specific methylase activity was quantified by adding 0.1 ml of cell extract to 7 μl of the following methylase reaction mixture: 0.015 mM SAM, 34 mM dithiothreitol, 0.5 μg of RNase A, 4 μg of bovine serum albumin, 5 μg of double-stranded GATC containing the DNA substrate (5′-CAGGATCCATGC GATCAACCGATCAAGGATCCAC-3′), and 0.55 μCi of [*methyl*-^{3}H]SAM. The reaction mixture was incubated for 1 h at 37°C, after which an excess of unlabeled SAM was added to reach a final concentration of 3.0 mM to stop the reaction. A 0.08-ml sample of each reaction mixture was transferred to Whatman DE81 filter paper and washed several times with 0.4 M NH_4HCO_3, followed by one wash with 95% ethyl alcohol. The filters were allowed to dry, and the amount of *methyl*-^{3}H incorporated onto the DNA substrate was determined by scintillation counting. Methylase activity in each cell extract was calculated as counts per minute of *methyl*-^{3}H per microgram of total protein in the cell extract.

Virulence, colonization, and protection assays. (i) LD_{50} assay. For *Yersinia*, an assay was used to determine the lethal dose required to kill 50% of the animals (LD_{50}); this virulence assay was performed as described in reference 20. Briefly, mutant and wild-type *Yersinia* species grown overnight in Luria broth at 28°C with shaking were washed in 0.15 M NaCl, diluted in 0.2 ml of 0.2 M phosphate buffer (pH 8.0), and used to perorally infect BALB/c mice by gastrointubation. The protective capacity of Dam derivatives was determined by challenging immunized mice with the virulent parental strain. Mice were examined daily following challenge for morbidity and mortality. To determine the number of bacteria in host tissues, moribund mice were sacrificed and bacteria were recovered from host tissues and plated for colony counts. Host tissues assayed included Peyer's patches (the four Peyer's patches proximal to the ileal-cecal junction), mesenteric lymph nodes, and spleens.

(ii) Competitive index assay. The competitive index is the ratio of mutant to wild-type organisms recovered from host tissue after infection. For *Y. pseudotuberculosis* infection, 6- to 8-week-old BALB/c mice were gastrointubated with 8.0×10^8 cells of mutant organisms and 8.0×10^8 cells of wild-type organisms. After 7 days, mice were sacrificed, spleens were recovered and homogenized, and bacteria were enumerated by direct colony count as described previously (5). For *V. cholerae* infection, 5-day-old CD-1 suckling mice were coinoculated perorally with approximately 10^5 mutant organisms and 10^5 wild-type organisms; 24 h postinfection, mice were sacrificed and bacterial numbers were isolated from the intestine as described previously (7).

Nucleotide sequence accession numbers. The sequence data for *V. cholerae dam* and *Y. pseudotuberculosis dam* have been submitted to the DDBJ, EMBL, and GenBank databases under accession numbers AF274317 and AF274318, respectively.

RESULTS

Characterization of *dam* from *Y. pseudotuberculosis* and *V. cholerae*. To clone the *dam* gene from *Y. pseudotuberculosis* and *V. cholerae*, recombinant plasmids derived from a genomic DNA library constructed from both pathogens were screened for the ability to complement the 2 aminopurine (2-AP) sensitivity phenotype of an *S. enterica* serovar Typhimurium *dam* mutant strain (2-AP is a purine analog which is toxic to *dam* mutants [14]). A 1.3-kb *Nco*I/*Pvu*II DNA fragment from *Y. pseudotuberculosis* conferred 2-AP resistance to a *dam* mutant serovar Typhimurium strain and encoded DNA adenine methylase activity as evidenced by resistance of the recombinant plasmid (recovered from *dam* mutant *E. coli*) to digestion with the restriction enzyme *Mbo*I, which cleaves only nonmethylated GATC sequences. Sequence analysis revealed an open reading frame (ORF) encoding a putative 271-amino-acid protein exhibiting 71% identity to the entire *E. coli* Dam protein. Taken together, these data indicate that this ORF encoded *Y. pseudotuberculosis* Dam activity.

A 1.2-kb *Eco*RI/*Dra*III *V. cholerae* DNA fragment conferred 2-AP resistance to *dam* mutant serovar Typhimurium and encoded DNA adenine methylase activity as judged by resistance of the plasmid clone to *Mbo*I cleavage. Sequence analysis of

the insert DNA revealed an ORF encoding a 277-amino-acid protein which displays 63.5% identity over the entire *E. coli* Dam protein. The *V. cholerae dam* gene described in this study differs from a previously published *V. cholerae dam* sequence, which partially overlaps and is in the opposite orientation to that of the *dam* gene identified here and has only 30 to 35% identity over the entire *E. coli* Dam sequence (1). Moreover, a recombinant plasmid containing the previously identified *dam* gene was unable to confer 2-AP resistance to a *dam* mutant serovar Typhimurium strain, and when this plasmid was recovered from *dam* mutant *E. coli*, it was completely digested by *Mbo*I, indicating the lack of DNA methylase activity (data not shown). These data suggest that the *dam* gene identified in this study encodes *V. cholerae* DNA adenine methylase activity that is specific for GATC sequences.

***dam* is essential for viability in *Y. pseudotuberculosis and V. cholerae*.** Standard genetic procedures to remove the *dam* gene from *Y. pseudotuberculosis* and *V. cholerae* were unsuccessful, suggesting that, in contrast to *Salmonella* and *E. coli, dam* is essential for viability in *Yersinia* and *Vibrio*. To confirm the requirement of *dam* for growth, suicide plasmids containing *dam* deletion mutations were integrated into the native chromosomal *dam* locus of *Y. pseudotuberculosis* and *V. cholerae*. The chromosome integration event generates a duplication of the *dam* locus, in which one copy is *dam*$^+$ and one copy has a mutation (22). Essentiality (on rich medium) was demonstrated by showing that the generation of the *dam* mutation haploid state from the parental *dam* duplication is dependent upon the presence of a *dam*$^+$ gene provided in *trans*. The *dam* deletion structure in the chromosomes of *Y. pseudotuberculosis* and *V. cholerae* was confirmed by both PCR and Southern analysis according to the method of Julio et al. (22). Table 2 shows that *dam* mutant segregants of *Y. pseudotuberculosis* and *V. cholerae* were obtained only in the presence of a wild-type copy of *dam* provided in *trans*. These data indicate that *dam* is essential for viability in *Y. pseudotuberculosis* and in *V. cholerae*, similar to the essential role of the cell cycle-regulated methyltransferase CcrM in *Caulobacter crescentus, Rhizobium meliloti, Brucella abortus*, and *Agrobacterium tumefaciens* (23, 33, 35, 44).

Dam overproduction attenuates the virulence of *Y. pseudotuberculosis* and *V. cholerae*. To examine whether altered levels of Dam activity affected the virulence of *Y. pseudotuberculosis* and *V. cholerae*, recombinant plasmids that overproduced *E. coli* Dam were introduced into both pathogens, and the resulting strains were assayed for virulence. Note that loss of the Dam-overproducing plasmid in a *dam*$^+$ parental background would result in a virulent (wild-type) strain. Thus, these virulence studies were performed with *dam* mutant parental backgrounds since *dam* is essential for viability in *Y. pseudotuberculosis* and *V. cholerae* and loss of the Dam-overproducing plasmids in *dam* mutant backgrounds is lethal for both pathogens.

Overproduction of *E. coli* Dam from a recombinant plasmid in *Y. pseudotuberculosis* (MT2294) and *V. cholerae* (MT2284), resulting in 74- and 53-fold increases in Dam activity, respectively, was not lethal but significantly attenuated the virulence of these two pathogens. That is, Dam overproduction results in a >6,000-fold attenuation in a *Y. pseudotuberculosis* murine bacteremia infection model and a 5-fold defect ($P < 0.05$) in *V. cholerae* colonization in a suckling mouse model (Table 3). The attenuation in both organisms was not due to a general growth defect since the Dam-overproducing strains showed growth rates in vitro similar to that of the wild type (data not shown). Relevant to these findings, CcrM overproduction was recently shown to attenuate the intracellular replication of *B. abortus* in murine macrophages (35).

TABLE 2. Dam is essential for viability in *Y. pseudotuberculosis* and *V. cholerae*[a]

Strain	Plasmid	No. of colonies with the indicated genotype at the *dam* locus after excision of pCVD442	
		dam$^+$	Δ*dam*
Y. pseudotuberculosis			
MT2357	None	200	0
MT2358	*dam*$^+$ (*Y. pseudotuberculosis*)	135	44
MT2359	*dam*$^+$ (*E. coli*)	151	21
MT2360	Vector alone	200	0
V. cholerae			
MT2361	None	196	0
MT2362	*dam*$^+$ (*V. cholerae*)	153	47
MT2363	*dam*$^+$ (*E. coli*)	118	76
MT2364	Vector alone	194	0

[a] Integration of the suicide plasmid pCVD442 (8) into the *Y. pseudotuberculosis* or *V. cholerae* chromosome results in a duplication of the *dam* locus wherein one copy contains the wild-type *dam* gene and the other copy contains *dam* with a deletion associated with a kanamycin resistance marker (22). Selection for the excision of pCVD442 on plates containing 10% sucrose result in either *dam*$^+$ or *dam* mutant (Δ*dam*::Kn) segregants (8). The *dam* mutant segregants of *Y. pseudotuberculosis* or *V. cholerae* are associated with chromosomal internal deletions of 314 or 324 bp of the *dam* sequence, respectively. For plasmid complementation experiments, pWKS30-Tc (43) was used to provide the *dam* gene from *Y. pseudotuberculosis, V. cholerae*, or *E. coli* as indicated.

Dam-overproducing *Y. pseudotuberculosis* ectopically secretes Yops. To understand the mechanism of *Yersinia* virulence attenuation, we questioned whether Dam-overproducing *Y. pseudotuberculosis* showed altered secretion of Yops. Yops, which play essential roles in *Yersinia* virulence, are normally under strict regulatory control by the low calcium response, whereby Yop secretion occurs in vitro only at 37°C under conditions of low calcium (40). However, overproduction of Dam in *Y. pseudotuberculosis* resulted in the relaxation of the temperature, but not the low-calcium, dependence of Yop secretion (Fig. 1). These data indicate that Dam participates in the environmental regulation of the secretion of *Yersinia* virulence proteins.

Dam-overproducing *Y. pseudotuberculosis* strains confer protective immune responses in mice. Because Dam-overproducing *Y. pseudotuberculosis* strains were attenuated for virulence, we determined whether they could serve as live attenuated vaccines against murine bacteremia. BALB/c mice were perorally immunized via gastrointubation with a dose of 9.3×10^9 cells of the *Y. pseudotuberculosis* Dam-overproducing strain MT2294, as described in footnote *a* of Table 2. Eight weeks later, the immunized mice were challenged perorally with 2.6×10^{10} wild-type *Y. pseudotuberculosis* cells. All (13 of 13) mice immunized with Dam-overproducing *Y. pseudotuberculosis* survived a wild-type *Yersinia* challenge of >1,000-fold above

TABLE 3. Dam overproduction confers a virulence defect in *Y. pseudotuberculosis* and *V. cholerae*

Strain	Relevant genotype[a]	Oral LD_{50} ratio[b] (mutant/wild type)	Competitive index[c]
MT2294	Dam^{OP} *Y. pseudotuberculosis*	>6,000	$<10^{-4}$
MT2284	Dam^{OP} *V. cholerae*	ND	0.218

[a] Bacterial strains are derivatives of *Y. pseudotuberculosis* strain YPIIIpYV and *V. cholerae* strain O395. Dam-overproducing (Dam^{OP}) strains of *Y. pseudotuberculosis* (MT2294) and *V. cholerae* (MT2284) contain *E. coli dam* on chloramphenicol- and tetracycline-resistant derivatives of the high-copy-number recombinant plasmid pTP166 (29) and the medium-copy-number plasmid pWKS30 (43), respectively, in Dam mutant (Δ*dam*::Kn) genetic backgrounds. (Dam overproduction from the high-copy-number plasmid pTP166-Cm in *V. cholerae* was deleterious to *V. cholerae* cell growth as evidenced by a 50% decrease in the rate of growth on rich medium.) Since *dam* is essential for viability in *Y. pseudotuberculosis* and *V. cholerae*, the loss of the Dam-overproducing plasmids in *dam* mutant backgrounds is lethal for both pathogens.

[b] The oral LD_{50} ratio (the LD_{50} of the Dam-overproducing strain divided by the LD_{50} of wild-type bacteria) was determined by infecting 18 BALB/c mice with 1.56×10^{11} cells of the *Y. pseudotuberculosis* Dam-overproducing strain MT2294 as described previously (20); 18 of 18 mice survived this challenge dose, and no visible signs of infection were observed. The peroral LD_{50} of wild-type *Y. pseudotuberculosis* (2.5×10^7) was determined by Monack et al. (30). ND, not determined.

[c] For *Y. pseudotuberculosis* infection, six BALB/c mice were gastrointubated with 8.0×10^8 *Yersinia dam* mutant cells containing the Dam-overproducing plasmid from strain MT2294 and 8.0×10^8 cells of the wild type. After 7 days, mice were sacrificed, spleens were recovered and homogenized, and bacteria were enumerated by direct colony counting as described previously (5). Of the 10^6 to 10^7 *Yersinia* organisms recovered from each of six spleens, none contained the Dam-overproducing plasmid. For *V. cholerae* infection, six CD-1 mice were gastrointubated with a 1:1 ratio of Dam-overproducing cells (MT2284) to wild-type cells; 24 h postinfection, mice were sacrificed and bacterial numbers were determined from the intestine as described previously (7). The attenuation conferred was significant according to the two-tailed Fisher exact test ($P < 0.05$).

the LD_{50}. None of 14 unimmunized mice survived the challenge dose. The protection conferred was significant according to the two-tailed Fisher exact test ($P < 0.05$). Mice immunized with the *Yersinia* Dam-overproducing strain were cleared of the vaccine between day 5 and day 21 and thus Dam-overproducing *Y. pseudotuberculosis* cells were not present at the time of challenge (S. M. Julio et al., submitted for publication). Moreover, vaccinated animals blocked the proliferation of virulent (Dam^+) *Yersinia* in Peyer's patches, mesenteric lymph nodes, and spleens and cleared these virulent bacteria by 21 days postinfection (Fig. 2). These data suggest that mice vaccinated with *Yersinia* Dam-overproducing strains hinder the proliferation of virulent *Yersinia* in mucosal and systemic tissues, similar to what has been observed in mice vaccinated with *Salmonella* Dam mutants (19).

DISCUSSION

DNA adenine methylase plays a pivotal role in many bacterial functions, including the replication, repair, transposition, and segregation of chromosomal DNA (26, 28). Additionally, in *Salmonella*, Dam is a global regulator of bacterial gene expression and plays a critical role in virulence, and mutants with altered levels of Dam activity elicit protective immune responses to murine typhoid fever (12, 20). Here we explored the role of Dam in the pathogenesis of two other enteric bacteria, *Y. pseudotuberculosis* and *V. cholerae*. In contrast to results of studies performed with *E. coli* and *Salmonella* (4, 28), Dam was found to be essential for viability in *Yersinia* and *Vibrio*. Dam overproduction attenuated the virulence of *Y. pseudotuberculosis* and *V. cholerae*, leading to, in *Yersinia*, a fully protective immune response in vaccinated hosts. Since mutations in Dam attenuate the virulence of several diverse pathogens, the role of DNA methylation in virulence may emerge as a common theme in bacterial pathogenesis.

Dam's essential role in the viability of *Y. pseudotuberculosis* and *V. cholerae*, which are members of the gamma subdivision of proteobacteria, parallels the essential role of CcrM (cell cycle-regulated methyltransferase) for the viability of several proteobacteria of the alpha subdivision, including *C. crescentus, R. meliloti, B. abortus*, and *A. tumefaciens* (23, 35, 39, 44). Both Dam and CcrM catalyze the transfer of a methyl group from SAM to the N-6 position of adenine at specific target sequences within DNA. However, the target sequences of these two enzymes are different: Dam methylates GATC sequences, and CcrM methylates GANTC sequences. Moreover, both the catalytic and SAM binding domains of Dam and CcrM are arranged in a different linear order. For these reasons, Dam and CcrM belong to different methyltransferase groups. Despite these differences, DNA adenine methylation may exert its effects on diverse bacteria via its role as a global regulator of gene expression. That is, Dam regulates many (>20) *Salmonella* genes that are specifically induced during infection (20), and CcrM autoregulates *ccrM* transcription and has been implicated in the regulation of a number of genes involved in normal cell cycle progression (33, 34). Thus, the role of DNA methylation in regulating gene expression may explain, in part, Dam's function in many cellular processes of diverse bacteria.

DNA methylation plays a role in the virulence of a wide range of pathogens, including *Salmonella* spp. and *B. abortus* (which causes fetal-calf abortion) via Dam and CcrM activity, respectively (20, 26, 35). Dam also plays a role in *Salmonella* invasion, M-cell cytotoxicity, and the secretion of *Salmonella*

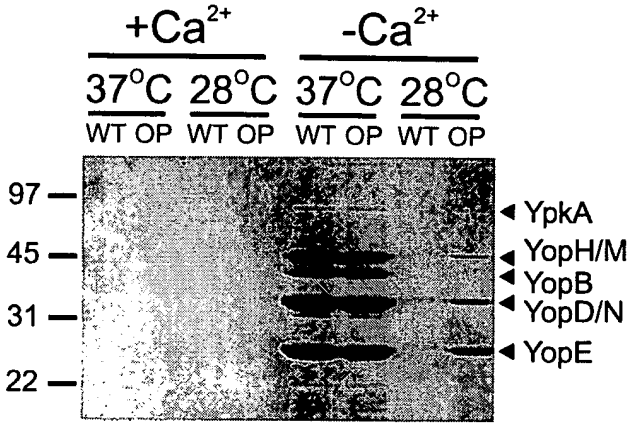

FIG. 1. Dam overproducer *Y. pseudotuberculosis* strains ectopically secrete Yops. Proteins were isolated from culture supernatants from wild-type (WT) and Dam overproducer (OP) *Yersinia* strains grown at the indicated temperatures in the presence ($+Ca^{2+}$) or absence ($-Ca^{2+}$) of calcium according to the method of Forsberg et al. (11). Cells were separated from the culture supernatant by centrifugation, supernatant proteins were precipitated with ammonium sulfate, and proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by Coomassie blue staining. Yops were identified according to the method of Hakansson et al. (16). Numbers refer to molecular weight standards (in thousands).

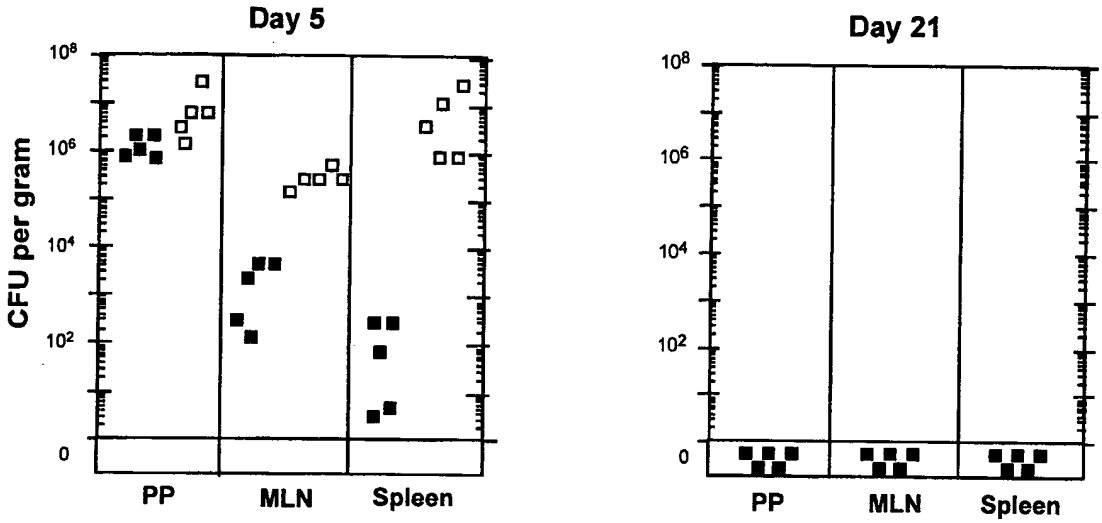

FIG. 2. Immunization with Dam overproducer *Yersinia* strains blocks the proliferation of virulent *Y. pseudotuberculosis* in mucosal and systemic tissues in mice. Virulent *Y. pseudotuberculosis* cells (3.2×10^9) were orally administered to nonvaccinated BALB/c mice (open boxes) or to mice vaccinated with 7.6×10^9 Dam overproducer *Y. pseudotuberculosis* cells (closed boxes). Vaccinated mice were orally challenged 8 weeks postimmunization. The fate of the virulent organisms was determined in host tissues at the times indicated after challenge. All nonvaccinated mice were dead by day 10. Mice immunized with the *Yersinia* Dam-overproducing strain were cleared of the vaccine between day 5 and day 21 and thus Dam-overproducing *Y. pseudotuberculosis* cells were not present at the time of challenge (Julio et al., submitted). PP, Peyer's patches; MLN, mesenteric lymph nodes.

virulence-associated proteins (12). Here we show that overproduction of Dam completely attenuated *Y. pseudotuberculosis* virulence and resulted in the export of Yops under conditions not normally permissive for secretion. Specifically, Dam overproduction relaxed the temperature dependence but not the low calcium dependence of Yop secretion, suggesting that Dam contributes to the strict environmental regulation governing the synthesis and/or secretion of *Yersinia* virulence proteins. Such altered protein secretion may attenuate the virulence of *Y. pseudotuberculosis*, as has been suggested for *Salmonella* (12). Moreover, the ectopic secretion of immunogens may contribute to the heightened immunity in hosts vaccinated with Dam mutants of *Yersinia* or *Salmonella* (19, 20).

The role of Dam in virulence and in the elicitation of protective immune responses may rely on its capacity as a global regulator of gene expression (19, 20, 24–26). Insights into the regulatory role of Dam have resulted from studies involving the *E. coli* pyelonephritis-associated pilus (*pap*) operon, which encodes pili that are required for infection of the urinary tract. The expression of *pap* genes is reversibly switched between the unexpressed state and the expressed state by a methylation-sensitive process termed phase variation (42). The reversible transition from non-pilus expressing to pilus expressing may allow the bacteria to attach and detach from urogenital tissues, enabling initial colonization and infection of the bladder and subsequent colonization and infection of the kidney, causing cystitis and pyelonephritis, respectively. Dam target sites (GATC sequences) in the *pap* promoter are protected from methylation by the binding of regulatory proteins at or near these sites, forming specific DNA methylation patterns analogous to what has been observed in eukaryotes (3, 17, 21, 41).

DNA methylation can modulate gene expression by altering the affinity of regulatory proteins for DNA, and, conversely, regulatory proteins can bind to nonmethylated Dam target sites, protecting these sites from methylation. Dysregulation of Dam activity can disable the ability of a pathogen to cause disease via aberrant virulence gene expression and contribute to the heightened immunity in vaccinated hosts through the ectopic production of an expanded repertoire of potential antigens. While a concern of this approach is that Dam overproducer strains can revert to wild-type virulence by mutation, insertion of multiple nontandem copies of Dam-overproducing cassettes in the chromosome should reduce the likelihood of this undesired scenario. Because the Dam methylase is essential for bacterial virulence or viability in multiple gram-negative pathogens (24, 26), Dam inhibitors are a promising target for antimicrobial drug development.

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